Supplementary Methods

Plasmids, siRNA, and antibodies

pEGFP-N1 containing p14ARF was obtained from FHGB (21C Frontier Human Gene Bank, Seoul, South Korea). p14ARF WT-3XFLAG/CMV and p14ARF deletion mutants (1-64 and 65-132)-3XFLAG/CMV were prepared by PCR. MKRN1 cDNA and its mutants were as reported previously (21-22). pEGFP-C2 used as a transfection control was from Clontech (San Diego, CA). MKRN1 siRNA #5 (5'-CAGGCGAAGCTGAGTCAAGAA-3'), #6 (5'-CGGGATCCTCTCCAACTGCAA-3'), #7 (5'-caggcgaagctgagtcaag-3'), p14ARF siRNA #1 (5'-GAACAUGGUGCGCAGGUUCTT-3') and control siRNA were made by Qiagen-Xeragon (Valencia, CA). MKRN1 shRNA #2 (TRCN0000033796), #5 (TRCN0000296491) and p14ARF shRNA (TRCN00000010482) were purchased from Sigma-Aldrich (St Louis, MO). Polyclonal rabbit anti-HA (Y-11) (1:1000 dilution), monoclonal mouse anti-HA (F-7) (1:1000 dilution), and polyclonal rabbit anti-GFP (FL) (1:5000 dilution) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal mouseanti-FLAG (M2) (1:2000 dilution), polyclonal rabbit anti-FLAG (1:2000 dilution), and monoclonal mouse anti-actin (1:5000 dilution) antibodies were obtained from Sigma-Aldrich. Polyclonal rabbit anti-MKRN1 antibody (1:1000 dilution) and polyclonal rabbit anti-p14ARF antibodies (A300-340A and A300-342A) (1:1000 dilution) were purchased from Bethyl Laboratories (Montgomery, TX). Monoclonal mouse anti-p14ARF antibody (ab-3) (1:1000 dilution) was from Labvision (Fremont, CA). Polyclonal rabbit anti-p19ARF antibody (ab80) (1:1000 dilution) was from Abcam (UK). Monoclonal mouse anti-p14ARF antibody (Cell Signaling) and polyclonal rabbit anti-MKRN1 antibody (Bethyl Laboratories) were used for

immunohistochemistry.

Cell culture

Human non-small cell lung carcinoma (H1299), embryonic kidney cell line expressing SV40 large T antigen (293T), cervical cancer cell line (HeLa), lung fibroblast (IMR90) and foreskin fibroblast (HFF) were obtained from American Type Culture Collection (ATCC, Manassas,VA) and grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% Fetal bovine serum. IMR90 and HFF cells of passage number 8 or less were used. All human gastric cancer cell lines including human stomach adenocarcinoma cell line (AGS) and gastric carcinoma cell lines (SNU-5, SNU-216, SNU638, SNU-668, SNU601, SNU-719, MKN28, YCC-2 and KATO-III) were purchased from the Korea Cell Line Bank (KCLB, Seoul, Korea) and were grown in RPMI medium supplemented with 10% fetal bovine serum. The ATCC and KCLB authenticate the phenotypes of these cell lines on a regular.

Cellular and biochemical analyses

For the crystal violet staining assay, cells were transfected with 30nM siRNA using Lipofectamine RNAiMAX three times, followed by one wash with PBS and fixation with 1% glutaraldehyde for 10 min. After washing with PBS, cells were stained with 0.5% crystal violet for 10 min at RT. For immunoprecipitation assays, cells were plated in 100-mm dishes and transfected with plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 24 hr of transfection, cells were lysed with Lysis buffer (50mM Tris-HCl (pH7.5), 150mM NaCl, 0.5% NP-40, 0.5% Triton X-100, 0.1% Na-deoxycholate, 1mM EDTA, and protease inhibitors). Lysates were immunoprecipitated with antibodies for 2 hr at 4°C and incubated

with protein G-sepharose beads (GE healthcare, Buckinghamshire, United Kingdom) for 2 hr at 4°C. Beads were centrifuged and eluted with 2X sample buffer. Immunofluorescence assays were performed as follows: Cells were plated in 12-well plates with cover slips. Transfection of plasmids was conducted using Lipofectamine 2000. After 24 hr transfection, cells were fixed in 10% paraformaldehyde for 10 min and then washed three times with PBS. The fixed cells were permeabilized by 0.5% Triton X-100 in PBS for 10 min and washed three times with PBS. The cells were blocked with 5% bovine serum albumin (BSA) in PBS for 30 min and incubated with primary antibodies in 2.5% BSA overnight. The cells were then washed three times for 5 min each prior to incubation with Alexa Fluor 488-conjugated anti-mouse or Alexa 594-conjugated anti-rabbit antibodies (Molecular Probes, Eugene, OR) for 1 hr. The cells were washed, stained with 4',6-diamidino-2-phenylindole (DAPI) for 10 min, washed again, mounted on slides, and analyzed using N-SIM super-resolution microscopy. For the cell viability assay, H1299, HeLa, AGS and SNU 601 cells were plated and cultured for 24, 48, 72 and 96 h. The cells were incubated with CellTiter-Glo® Reagent (Promega, G7571, USA) for 15 min at 37°C. Luminescence was recorded using a luminometer.

RT-PCR

Total RNA was subjected to RT-PCR using p14ARF primers, 5’-cgg aat tca tgg tgc gca ggt tct tg-3’ (forward) and 5’-cg gaa ttc ttt ggt ctt cta gga agc gg-3’ (reverse). MKRN1 primers and PCR method were as reported previously (21).

Preparation of MKRN-1 shRNA loaded lentivirus and stable AGS cell lines

We prepared stable AGS cell lines with reduced MKRN1 and/or p14ARF expression by interference with specific shRNAs. Lentiviral vectors containing MKRN1 or p14ARF shRNA sequences were purchased from Sigma-Aldrich. The lentivirus particles were generated by co-transfection of 293FT cells with lentiviral vectors and three plasmids, VSVG, RSV-REV, and PMDLg/pPRE, using Lipofectamine 2000 transfection reagent following the manufacturer's instructions (Invitrogen). Two days after transfection, the cell culture media was filtered using a 0.45-μm filter. AGS cells were transfected with these lentiviral particles and sorted using puromycin. MKRN1 and p14ARF protein and mRNA expression levels were determined by Western blot and RT-PCR analysis, respectively.

Ubiquitination assay

Ubiquitination assays were performed under denaturation conditions using Ni^{2+}-nitrilotriacetic acid (NTA) beads, as reported previously (22) as well as by immunoprecipitation of HA/Ub. After the denaturation of proteins in cells by SDS and lysis by lysis buffer (50mM Tris-HCl (pH7.5), 150mM NaCl, 0.5% NP-40, 0.5% Triton X-100, 0.1% Na-deoxycholate, 1mM EDTA, and protease inhibitors), cell lysates were immunoprecipitated with anti HA antibody.

SA- β-galactosidase assay

The β-galactosidase assay_for senescence was performed using a senescence detection kit (#K320-250, BioVision, Mountain View, CA). Briefly, cells were plated in 60-mm dishes and cultured for 2-3 days, then washed once with phosphate buffered saline (PBS) and fixed with fixation solution for 15 min at RT. Cells were washed two times with PBS and incubated with

staining solution overnight at 37°C, followed by microscopic analyses.

Preparation of MKRN1 null MEFs

FVB/NJxC57BL/6J F2 mice with a MKRN1-knockout allele (heterozygous mice, +/-) were kindly provided by Drs. Deyu Feng and T.A. Gray (Northwestern University and David Axelrod Institute) (23). $MKRN1^{+/-}$ mice were further backcrossed until N6 in the B6 background. Female and male N6 $MKRN1^{+/-}$ mice were crossed to produce $MKRN1^{+/+}$, $MKRN1^{-/-}$ MEFs. To obtain MEFs from 13.5 day old embryos, embryos were washed using PBS (Welgene) and minced. After mincing, MEFs were incubated with 3ml trypsin/EDTA (Gibco) on 37℃ for 15min. Trypsinized MEFs were transferred to 150mm culture dishes and 20ml DMEM containing 10% fetal bovine serum (FBS, GIBCO) was added. The dishes were incubated at 37℃ for 4-8hr. After 4-8hr, media were exchanged, MEFs were retained in DMEM containing 10% FBS and were subcultured at a 1:3 ratio upon reaching confluence.

***In vivo* gastric cancer xenografted mouse models**

All animal experiments were approved by the Institutional Review Board of National Cancer Center (NCC Korea) and performed under specific pathogen-free facilities and conditions in accordance with the Guidelines for the Care and Use of Laboratory Animals of NCC (NCC-11-034D). Six-week-old female specific pathogen free Balb/c nude mice were purchased from Central Lab (Animal Inc., Korea). Mice were inoculated subcutaneously (s.c.) into both flanks with 10^6 cells in each flank of stable AGS and SNU601 cells under 20 μl of ketamine/rompun (9:1) anesthesia. From palpable tumor formation until termination, tumor sizes were measured every 2 or 3 days using calipers and tumor volume was calculated

according to the formula, length x width^2 x 0.5236. Mice were killed in 7.5% CO_2 chamber and tumors were harvested for immunohistochemical and other analyses.

Human gastric cancer Tissue Micro-Array analyses

For immunohistochemical analysis, core tissue biopsy specimens (2 mm diameter) were obtained from individual paraffin-embedded gastric carcinomas (donor blocks) and arranged in new recipient paraffin blocks (tissue array blocks) using a trephine apparatus (Superbiochips Laboratories, Seoul, South Korea). Immunohistochemical analysis of MKRN1 and p14ARF was performed as described previously (24).